Longitudinal Changes in Corneal Cell and Nerve Fiber Morphology in Young Patients with Type 1 Diabetes with and without Diabetic Retinopathy: A 2-Year Follow-up Study

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Purpose. We have previously used in vivo corneal confocal microscopy (IVCCM) to demonstrate significant alterations in the corneal epithelial cells, stromal keratocytes, and subbasal nerves in young patients with type 1 diabetes mellitis (T1DM), especially those with diabetic retinopathy (DR). We have evaluated the change in corneal cellular and subbasal nerve morphology over 2 years in young patients with T1DM with or without DR.

Methods. A total of 19 patients with T1DM, without ($n = 12$) and with ($n = 7$) DR and 19 age- and sex-matched healthy control subjects underwent quantification of corneal cellular and subbasal nerve plexus morphology by using IVCCM at baseline and after 2 years.

Results. There was no significant change in corneal basal epithelial, posterior stromal keratocyte, or endothelial cell densities over 2 years. However, there was a significant reduction in corneal nerve branch ($P = 0.03$) and total nerve branch density ($P = 0.04$) in patients without DR and a significant reduction in corneal nerve fibre density ($P = 0.004$) in those with DR.

Conclusions. IVCCM can detect a progressive loss of corneal nerve fibers in young patients with T1DM and may allow the identification of individuals at risk of neuropathy progression for more active risk factor reduction.

Keywords: corneal confocal microscopy, type 1 diabetes mellitus, follow-up

Type 1 diabetes mellitus (T1DM) occurs in around 10% of all people with diabetes, affecting 20 million people worldwide, and the prevalence of newly diagnosed cases is predicted to increase by 0.24% to 0.30% over the next 5 to 15 years.[1] Microvascular complications can occur in young people with T1DM,[2–4] and diabetic retinopathy (DR) is considered to be the earliest and most common complication.[5,6]

Optical coherence tomography (OCT) has shown reduced thickness of the retinal nerve fiber and ganglion cell layers in children with T1DM without retinopathy, which is suggestive of early neuronal damage.[6–8] Furthermore, in vivo corneal confocal microscopy (IVCCM), a rapid noninvasive ophthalmic imaging technique has also been shown to identify early corneal cellular and nerve fiber pathology in children and adolescents with T1DM,[9] adults with T1DM without neuropathy,[10] or retinopathy or microalbuminuria.[11] Furthermore, reduced corneal nerve fiber length predicts the development of clinical diabetic neuropathy[12] and the development or worsening of retinopathy.[13] This early corneal nerve fiber damage has been attributed to elevated hemoglobin A_{1c} (HbA_{1c}) and triglycerides and a lower high-density lipoprotein (HDL).[14] Corneal nerve fiber regeneration has been observed after combined pancreas and kidney transplantation[15] and after treatment with the novel nonerythropoietic peptide ARA 290.[16] Based on our previous study showing reduced corneal nerve fiber parameters in young adolescents with T1DM with and without DR,[9] the present case-control follow-up study assessed the progression of corneal cellular and nerve fiber abnormalities in these young T1DM patients.

Methods

Study Subjects

All subjects were attending the Ophthalmology Department, Faculty of Medicine, University of Debrecen. Ethical approval was obtained from the University of Debrecen Ethics Committee (number 4701A-2016), and all subjects provided written informed consent in accordance with the Declaration of Helsinki. In case of children under 18 years of age, written informed consent was obtained from their parents or legal guardians.

At baseline, DR status was graded in T1DM patients according to the International Clinical Diabetic Retinopathy Disease Severity Scale by using fundus photography. Patients were classified into no DR (NDR) and DR groups and compared with age- and sex-matched healthy volunteers (CNDR and CDR) without diabetes or history of systemic inflammatory or ocular

Table 1. Demographics of Control Subjects and Patients with T1DM

	Groups					
	CNDR, $n = 12$	Baseline NDR, $n = 12$	Follow-up NDR, $n = 12$	CDR, $n = 7$	Baseline DR, $n = 7$	Follow-up DR, $n = 7$
Age, y						
Mean	17	14	16	37	34	36
±SD	4	3	3	6	6	6
Sex						
Male	4	4	4	6	6	6
Female	8	8	8	1	1	1

CNDR, controls for T1DM patients without DR (NDR); CDR, controls for T1DM patients with DR expressed as mean ± SD.

diseases. Exclusion criteria included contact lens wear and previous intraocular surgery.

Clinical and Ophthalmologic Investigations

Participants with T1DM underwent a comprehensive medical examination that included measurements of HbA_{1c}, serum lipids, estimated glomerular filtration rate (eGFR), blood pressure, and microalbuminuria. Ophthalmologic examination included slit-lamp examination, dilated fundus photography, intraocular pressure measurement, and IVCCM. Additional neuropathy tests and tear layer assessments were not performed.

In Vivo Corneal Confocal Microscopy

In 2014, we undertook IVCCM in 28 young patients with T1DM, with ($n = 10$) and without ($n = 18$) DR.[9] Nineteen of these T1DM patients with ($n = 7$) and without ($n = 12$) retinopathy who had continued to be seen by their diabetologist under standard clinical care, agreed to be reassessed in 2016. IVCCM analysis was performed using the Heidelberg Retina Tomograph III Rostock Cornea Module (HRT III RCM; Heidelberg Engineering GmbH, Heidelberg, Berlin, Germany). Local anesthetic (tetracaine hydrochloride 0.5%) was applied, and the subject was asked to focus on a distant target before scanning the central cornea. The right eye of normal controls and NDR patients was used for analysis, and in patients with DR, only the eligible eye (no previous intraocular surgery) was used for analysis. Section and volume scans were recorded from the basal epithelium anterior to Bowman's layer, subbasal nerve plexus (SBP), posterior stroma anterior to Descemet's membrane and endothelial cells (volume scans from the epithelium to the endothelial cells, and good quality section scans at the missing depths).

Image Selection

Three representative images of good quality were chosen from the basal epithelium, SBP, posterior stromal (keratocytes), and endothelial cell layers. A region of interest (ROI) was chosen from each cell layer containing at least 50 cells.

Image Analysis

The ROI area was 0.003 ± 0.007 mm^2 for the epithelium, 0.100 ± 0.009 mm^2 for the stromal keratocyte layer, and 0.028 ± 0.009 mm^2 for the endothelium. Focus position was 7 ± 3 μm for the epithelium, 65 ± 11 μm for the keratocytes, and 564 ± 17 μm for the endothelium. The cells were marked manually and the instrument-based software (Heidelberg Eye Explorer software; Heidelberg Engineering GmbH, Heidelberg, Berlin, Germany) automatically calculated cell densities (cells/mm^2). SBP morphology was quantified using automated software (ACCMetrics version 2.0; University of Manchester, Manchester, UK).[17] The following parameters were quantified: corneal nerve fiber density (CNFD), the number of nerve fibers/mm^2; corneal nerve branch density (CNBD), the number of primary branch points on the main nerve fibrers/mm^2; corneal nerve fiber length (CNFL), the total length of nerves mm/mm^2; corneal nerve fiber total branch density (CNTBD), the total number of branch points/mm^2; corneal nerve fibre area (CNFA), the total nerve fibre area mm^2/mm^2; and corneal nerve fiber width (CNFW), the average nerve fiber width mm/mm^2.

Statistical Analysis

The statistical analysis was performed using SPSS (version 22.0) and MedCalc (version 18.2.1) statistical programs for Windows. Descriptive data are shown as mean ± standard deviation (SD) and 95% confidence interval (CI). Student's *t*-test was used to determine the differences between patients with T1DM and controls. A paired *t*-test was used to compare corneal cellular and nerve morphology between baseline and follow-up. For univariate analysis, the χ^2 test and for bivariate datasets Pearson correlation test was used. Case-control analysis was performed with statistical significance criterion set at $P < 0.05$ in all cases.

Results

Demographic and Clinical Data

The demographic data are summarized in Table 1, and the clinical metabolic data of T1DM patients with and without DR are shown in Table 2. Total cholesterol decreased ($P = 0.04$) in the DR group, while triglycerides, HDL cholesterol, eGFR, blood pressure, and microalbuminuria showed no significant change over 2 years.

Corneal Cell Densities

There was no significant change in epithelial, keratocyte and endothelial cell densities, and central corneal thickness in patients with T1DM with and without DR compared to control subjects (Table 3; Fig. 1, 2).

Corneal SBP

There was no significant difference in any corneal nerve parameter between T1DM patients without DR and control subjects. CNFL was significantly lower in T1DM patients with DR compared to control subjects ($P = 0.002$). Over 2 years,

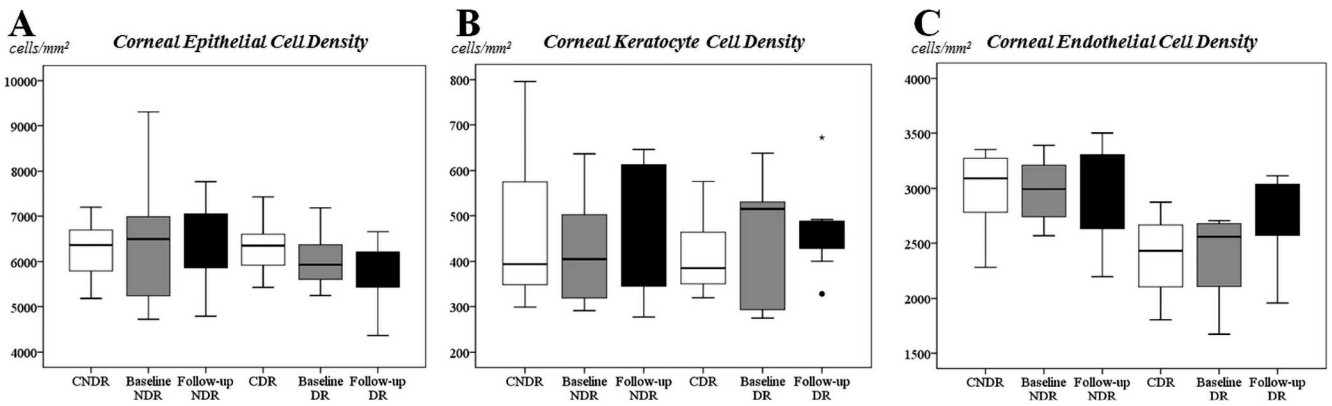

Figure 1. Corneal cell densities in controls compared to T1DM over 2 years. Patients with T1DM without (NDR) and with (DR) retinopathy compared to controls (CNDR and CDR) at baseline and follow-up. The basal epithelial cell (**A**), keratocyte (**B**), and endothelial cell (**C**) densitites measured with in vivo corneal confocal microscopy (IVCCM). Controls (*white bars*), T1DM baseline (*gray bars*), and follow up (*black bars*). Box and whiskers show the mean values with interquartile range.

there was a significant decrease in CNBD ($P = 0.03$) and CNTBD ($P = 0.04$), with a trend for reduction in CNFA ($P = 0.08$) and CNFW ($P = 0.07$) in the NDR group (Fig. 3). There was a significant reduction in CNFD ($P = 0.04$) in the DR group (Table 4).

Correlation Analysis

In patients with T1DM, serum triglycerides correlated inversely with CNFW ($r = -0.339$, $P = 0.04$), but there was no significant correlation between the duration of diabetes, HbA_{1c}, serum triglycerides, HDL, and eGFR with any other corneal nerve parameters ($P > 0.05$).

Discussion

IVCCM is a rapid, noninvasive ophthalmic imaging technique that detects subclinical nerve damage[18] and predicts the development of neuropathy in adults with diabetes.[12,19] In two large pooled analyses, a normative age range[20] and a good diagnostic ability for diabetic peripheral neuropathy have been established.[21] A reduction in basal epithelial and intermediate cell density was related to diabetes duration and diastolic blood pressure, while reduced CNFD and CNFL were related to HbA_{1c} in adults with T1DM and type 2 diabetes mellitus (T2DM).[22] Additionally, CNFD, CNDB, and CNFL were reduced in adults with T2DM with and without DR, while epithelial,

Table 2. Clinical and Metabolic Data of T1DM Patients With (DR) and Without (NDR) Retinopathy, at Baseline and Follow-up

	Baseline NDR, $n = 12$	Follow-up NDR, $n = 12$	P^*	Baseline DR, $n = 7$	Follow-up DR, $n = 7$	P^*
Duration of DM, y						
Mean	6	9		22	24	
±SD	3	3		7	7	
HbA_{1c}, %			0.278			0.894
Mean	7.82	8.14		8.29	8.43	
±SD	1.09	1.22		0.93	2.05	
95% CI	7.1–8.5	7.4–8.9		7.4–9.2	6.5–10.3	
Cholesterol, mmol/L			0.123			0.042
Mean	4.03	4.37		5.23	5.10	
±SD	0.51	0.73		0.59	1.35	
95% CI	3.7–4.4	3.9–4.9		4.3–6.2	1.7–8.5	
Triglycerides, mmol/L			0.067			0.317
Mean	0.83	0.73		2.43	1.45	
±SD	0.27	0.18		1.80	0.78	
95% CI	0.7 to 1.0	0.6 to 0.9		−2.0 to 6.9	−5.5 to 8.4	
HDL cholesterol, mmol/L			0.320			0.578
Mean	1.73	1.84		1.93	1.85	
± SD	0.25	0.28		1.21	0.07	
95% CI	1.6 to 1.9	1.7 to 2.0		−1.1 to 4.9	1.2 to 2.5	
eGFR, mL/min/1.73m^2			0.801			0.943
Mean	>90 in all patients	>90 in all patients		81.71	80.71	
±SD				21.92	24.57	
95% CI				61.4 to 101.9	57.9 to 103.4	
Hypertension	0	0		2	2	
Insulin pump users	5	5		0	0	
Microalbuminuria	0	0		2	2	

* P values, paired t-test to determine the differences between baseline and follow-up.

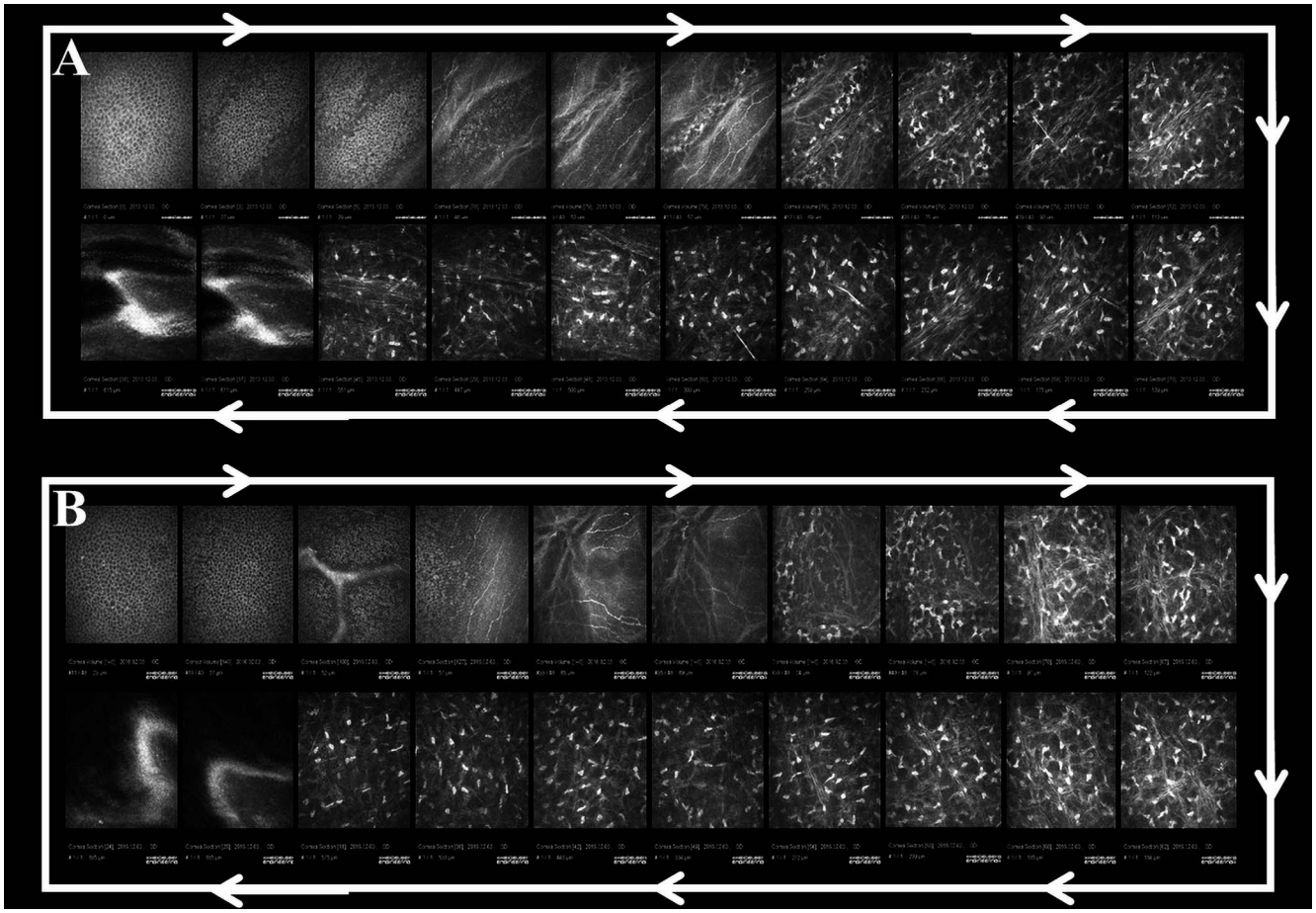

Figure 2. Corneal cell identification using Heidelberg Retina Tomograph instrument-based software. Section and volume scans from the corneal cell layers in the same patient's same eye with T1DM with DR at follow-up, at baseline (**A**) and after 2 years (**B**).

Table 3. Corneal Cell Densities and Central Corneal Thickness in Patients with T1DM Compared to Control Subjects at Baseline and Follow-up

	Groups					
	CNDR, $n = 12$	**Baseline NDR, $n = 12$**	**Follow-up NDR, $n = 12$**	**CDR, $n = 7$**	**Baseline DR, $n = 7$**	**Follow-up DR, $n = 7$**
Epithelial cell density, cells/mm^2						
Mean	6,248.33	6,333.72	6,514.74	6,327.58	6,048.71	5,743.95
±SD	639.62	1,290.25	877.19	650.76	671.17	772.87
95% CI	5,818.6–6,678.0	5,513.9–7,153.5	5925.4–7104.1	5,783.5–6,871.6	5428.0–6669.5	5,029.2–6,458.7
*P**†	0.845*	0.701†		0.894*	0.446†	
Keratocyte cell density, cells/mm^2						
Mean	475.73	414.62	466.40	385.00	439.38	472.40
±SD	168.97	115.21	140.48	70.14	148.27	105.18
95% CI	362.2–5,889.2	337.2–492.0	372.0–560.8	311.4–458.6	302.3–576.5	375.1–569.7
P	0.334*	0.356†		0.668*	0.639†	
Endothelial cell density, cells/mm^2						
Mean	2,977.95	2,980.48	2,980.70	2,384.50	2,343.53	2,652.37
±SD	406.53	292.12	490.10	442.18	445.26	464.62
95% CI	2,551.3–3,404.6	2,736.3–3,224.7	2,604.0–3,357.4	1,680.9–3,088.1	1,790.6–2,896.4	2,075.5–3,229.3
P	0.989*	0.999†		0.429*	0.315†	
Central corneal thickness, µm						
Mean	551.54	562.067	560.00	545.06	560.46	576.86
±SD	28.21	25.48	34.89	20.21	23.55	33.28
95% CI	532.6–570.5	545.8–578.3	538.4–582.7	528.1–562.0	538.7–582.2	546.1–607.6
P	0.358*	0.907†		0.196*	0.308†	

CNDR, controls for NDR group; CDR, controls for DR group.

* *P* value determined using Student's *t*-test to compare the baseline T1DM groups with controls.

† *P* value determined using paired *t*-test to determine the difference between baseline and follow-up.

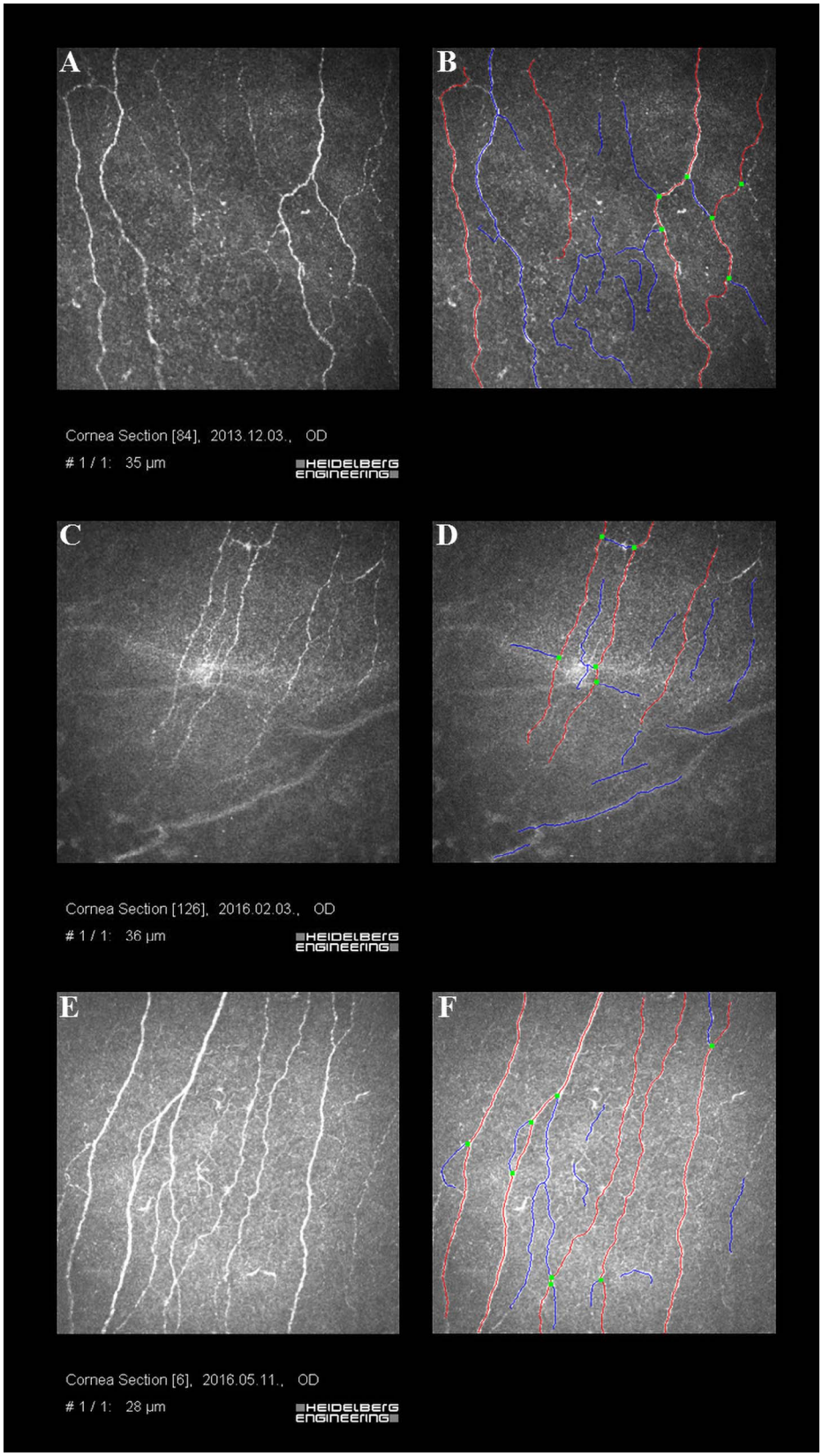

FIGURE 3. Original and annotated representative images of the SBP by using ACCMetrics software. SBP morphology of a patient with T1DM with DR at baseline (**A**, **B**) and after 2 years (**C**, **D**) compared with a healthy age- and sex-matched subject (**E**, **F**). *Red lines* (main fibers), *blue lines* (branches), and *green points* (branch points) showing reduced corneal nerves at baseline with a further reduction at follow up.

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Table 4. SBP Morphology in Control Subjects and Patients with T1DM at Baseline and Follow-up

	Groups					
	CNDR, $n = 12$	**Baseline NDR, $n = 12$**	**Follow-up NDR, $n = 12$**	**CDR, $n = 7$**	**Baseline DR, $n = 7$**	**Follow-up DR, $n = 7$**
CNFD, no./mm^2						
Mean	13.97	10.81	10.76	16.93	14.58	6.60
±SD	7.36	11.09	9.26	4.45	7.36	3.59
95% CI	9.0–18.9	0.5–21.1	1.0–20.5	13.2–20.7	5.4–23.7	2.8–10.4
P	0.530*	0.173†		0.485*	0.043†	
CNBD, no./mm^2						
Mean	12.59	12.36	6.25	16.66	16.25	6.25
±SD	7.48	9.50	5.43	5.59	12.79	5.43
95% CI	7.6–17.6	3.6–21.2	0.5–11.9	11.9–21.3	0.4–32.1	0.5–11.9
P	0.196*	0.029†		0.936*	0.114†	
CNFL, mm/mm^2						
Mean	10.33	10.67	8.58	12.52	8.43	7.71
±SD	3.19	4.98	3.49	0.85	2.96	1.44
95% CI	8.2–12.5	6.1–15.3	6.2–10.9	11.8–13.2	5.7–11.2	6.4–9.1
P	0.494*	0.153†		0.002*	0.573†	
CNTBD, no./mm^2						
Mean	28.50	28.82	20.36	31.44	21.73	20.83
±SD	12.93	14.44	10.67	10.64	13.28	12.15
95% CI	19.8–37.2	15.5–42.2	13.2–7.5	22.5–40.3	9.5–34.0	9.6–32.1
P	0.214*	0.035†		0.140*	0.898†	
CNFA, mm^2/mm^2						
Mean	0.0047	0.0057	0.0047	0.0053	0.0046	0.0051
±SD	0.0020	0.0013	0.0012	0.0001	0.0017	0.0019
95% CI	0.003–0.006	0.005–0.007	0.004–0.006	0.005–0.006	0.003–0.006	0.003–0.007
P	0.160*	0.086†		0.404*	0.694†	
CNFW, mm/mm^2						
Mean	0.0235	0.0240	0.0260	0.0220	0.0240	0.0250
±SD	0.0028	0.0031	0.0038	0.0019	0.0031	0.0044
95% CI	0.022–0.025	0.021–0.027	0.023–0.028	0.021–0.024	0.021–0.028	0.021–0.028
P	0.839*	0.074†		0.239*	0.760†	

CNDR, controls for T1DM without NDR; CDR, controls for T1DM with DR.

* *P* values determined with Student's *t*-test to compare baseline T1DM to controls.

† *P* values determined with paired *t*-test to determine the difference between baseline and follow-up.

stromal, and endothelial cell densities were only reduced in patients with DR.[23] Furthermore, in T1DM we have shown an early reduction in corneal nerve fibers in patients without DR or microalbuminuria.[24]

IVCCM can be undertaken reliably and with good reproducibility in children with T1DM.[25] An initial study in children and adolescents with T1DM showed no difference in corneal nerve morphology.[26] Although, more recently we have shown a reduction in epithelial and endothelial cell densities and a reduction in CNDB and CNFL in adolescent T1DM patients without DR and a further reduction in CNFD and CNBD in those with DR.[9]

There are no longitudinal studies assessing change in corneal cellular morphology, and only one previous study has assessed nerve fiber morphology over time in adults with T1DM, showing a reduction in CNFD and CNBD, which was related to HbA_{1c}.[14] This is the first longitudinal study in young adolescents with T1DM and it shows no significant change in epithelial, stromal, and endothelial cell densities over 2 years. With regard to early nerve fiber alterations, previous studies have shown a reduction in corneal and retinal nerve fiber parameters prior to the development of retinopathy[13,27] and a relatively greater reduction in CNFL in patients with T1DM compared to T2DM.[28] In the present study over 2 years, there was a significant decrease in the distal branches (CNBD and CNTBD) in young patients with T1DM without DR and a reduction in more proximal nerves (CNFD) in patients with DR. This is consistent with a retrograde process of neurodegeneration that has been demonstrated recently with a greater reduction in the inferior whorl compared to central CNFL.[29] The diagnostic ability of IVCCM may be further enhanced by assessing the inferior whorl or using wide-area mosaics, which take into account both proximal and distal corneal nerve morphology.[30,31]

Multiple pathogenetic mechanisms, including advanced glycation, increased flux through the sorbitol pathway, and oxidative stress, have been implicated in the development of diabetic neuropathy.[32] Experimental studies using a combination of menhaden oil, α-lipoic acid, and enalapril have been shown to ameliorate oxidative and inflammatory stress and improve distal corneal nerve morphology.[33] Several recent studies have shown early corneal nerve fiber regeneration in T1DM patients after combined pancreas and kidney transplantation,[15] T2DM patients treated with the novel nonerythropoietic peptide ARA 290,[16] and T1DM patients treated with omega-3 polyunsaturated fatty acid supplementation.[34] Furthermore, a recent study in patients with T2DM has shown that multifactorial intervention that reduces HbA_{1c}, blood pressure, and weight results in corneal nerve fiber regeneration.[35] Indeed, a change in corneal nerve morphology may prove to be a more sensitive end-point to assess the benefits of risk factor reduction on microvascular complications, given that the recent Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial showed no change in albumin excretion and

retinopathy in children treated with a statin and ACE inhibitor over 4 years.[36]

A limitation of the current study is the relatively small number of patients assessed at follow-up. However, this is the first longitudinal study of young adolescents with T1DM and it shows an early and progressive reduction in corneal nerve morphology. We believe these data support the potential utility of corneal nerve morphology quantification to assess the benefits of new therapies for diabetic neuropathy.

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